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Abstract

Telomerase activity is associated with over 90% of human breast cancers and is necessary for continued tumor cell growth, making it an ideal target for inhibition therapy. However, pharmacologic inhibitors of telomerase have not been as effective as expected. As such, our objective here is to identify novel telomerase interacting proteins and define their functional relationship to telomerase in order to provide additional targets for telomerase inhibition in breast cancer. The discovery of additional telomerase interacting proteins will be important for understanding the regulation of the enzyme, as well as provide additional targets for direct and indirect inhibition of telomerase to treat breast cancer.

We have generated normal and tumorigenic mammary-derived cell lines that over-express telomerase to serve as tools for identifying telomerase-associated proteins. Matched cell lines (with and without telomerase over-expressed) were lysed, immunoprecipitated using hTERT antibodies, and electrophoresed on a 1-D gel. Unique protein bands were excised from the gel (proteins that appeared in the telomerase over-expressors and not the control cells), and after purification, proteins were subjected to mass spectrometry (MALDI-TOF). The sensitivity of this initial experiment was too low for identifying the differentially precipitated proteins. To increase sensitivity, matched cell lines will be crosslinked to maintain protein:protein interactions, appropriately lysed, and immunoprecipitated with telomerase antibodies, and their protein expression profiles will be compared after 2-D gel electrophoresis. Differentially identified spots (those found in the telomerase over-expressing cells) will be excised, tryptically digested, and analyzed using mass spectrometry. Tryptic protein profiles will be identified and tested for their ability to interact with telomerase in our isogenic MCF-7 cells, as well as other mammary tumor cell lines (ZR-75, T-47D, and MDA MB231). We will also compare this association using a panel of non-mammary cancer cell lines to determine if there is breast specificity for these proteins. Thus, all of our proteomically identified telomerase interactors will be assessed for their biologic relevance and their ability to modulate the function of telomerase in mammary cells. It is expected that the biologically relevant proteins identified will provide additional targets for directed telomerase inhibition as a means to treat breast cancer, which is critical for understanding the role of telomerase during cancer progression.

Table of Contents

Introduction

Telomerase is a cellular reverse transcriptase that is associated with over 90% of human breast cancers and is composed of 2 integral components, an RNA template (hTR - human Telomerase RNA) and a catalytic polymerase (hTERT - human TElomerase Reverse Transcriptase) (Weinrich et al 1997). Telomerase is an obvious chemotherapeutic target (Shay and Bacchetti, 1997). Telomerase activity requires its two core components, hTERT and hTR, to be assembled into a functionally active enzyme by the Hsp90 chaperone complex (Holt et. al., 1999). We have previously demonstrated that chaperones are essential for optimal telomerase assembly *in vitro* (Holt et. al., 1999) and that Hsp90 itself remains associated with the functional telomerase complex (Forsythe et. al., 2001) (see Figure 1).

In a human cancer progression model, increased assembly of telomerase by chaperones, including Hsp90, has been shown to correlate with cancer progression, which is defined as increased aggressiveness *in vivo* (Akalin et. al., 2001). These findings indicate that increased expression of the Hsp90 chaperone complex with the associated activation of telomerase activity may be important steps in cancer formation (Holt et. al., 1999; Akalin et. al., 2001). While telomerase in cancer progression has been widely studied (reviewed by Shay and Bacchetti, 1997), the role of chaperones in carcinogenesis and their interplay between telomerase and its substrate, the telomere, are less well defined.

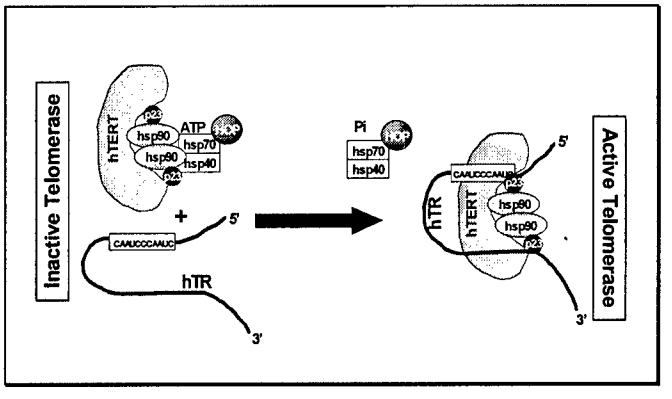

Figure 1. The hsp90 complex is required for assembly of active telomerase. Our working model for the chaperone-mediated ordered assembly of active human telomerase. [hTR - human telomerase RNA; hTERT - human telomerase reverse transcriptase]

Body

Rationale: The overall goal of this proposal is to identify mammary-specific telomerase interacting proteins that will serve as targets for direct and indirect inhibition of telomerase and determine the consequences of these interactions during breast cancer progression. Because human telomerase is associated with over 85% of all human malignancies and over 90% of malignant breast cancer, understanding the regulation of this enzyme is vital to determining novel and creative screening protocols and treatments. To aid in the study of telomerase regulation, we have established 2 independent mammary-related model systems to functionally analyze and identify additional regulatory proteins associated with telomerase. Using normal mammary epithelial cells and breast tumor-derived cells, we have over-expressed hTERT and have shown an increase in telomerase activity and telomere length in both cases (Figure 2 and Figure 3). Using these breast model systems, results generated from our proposal will identify mammary-specific telomerase interacting proteins and assess the importance of the interaction in terms of telomerase regulation and function in mammary-derived cells. Our proposed proteomic experimentation represents an innovative approach toward the discovery of proteins associated

with telomerase, and identification of proteins using mass spectrometry will be validated by standard molecular and cellular techniques to assess the functional and biological significance of the interaction. The proposed experiments will allow further understanding and characterization of the mechanisms of telomerase structure and function as it relates specifically to breast cancer and will facilitate innovative techniques and protocols for early detection and treatment of breast cancer.

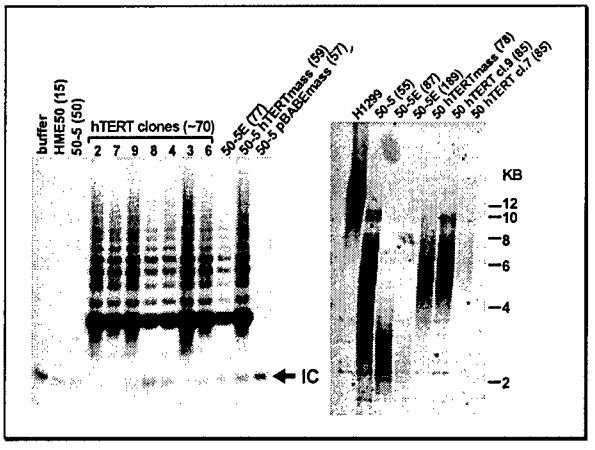

Figure 2. Ectopic expression of hTERT in HME cells results in activation of telomerase and an increase in average telomere length. (Left panel) The telomere repeat amplification protocol (TRAP) was used to show telomerase activity in exogenously expressing HME50 cell strains. The laddering effect is indicative of processive telomerase activity. The 36-bp internal control (IC) serves to normalize sample variation. (Right panel) A terminal restriction fragment (TRF) assay measures telomere length. Radiolabeled (TTAGGG)4 is hybridized in-gel to digested DNA. Heterogeneous smears are indicative of telomere length ranges. Numbers in parentheses represent population doubling (PD) at cell lysis or DNA isolation.

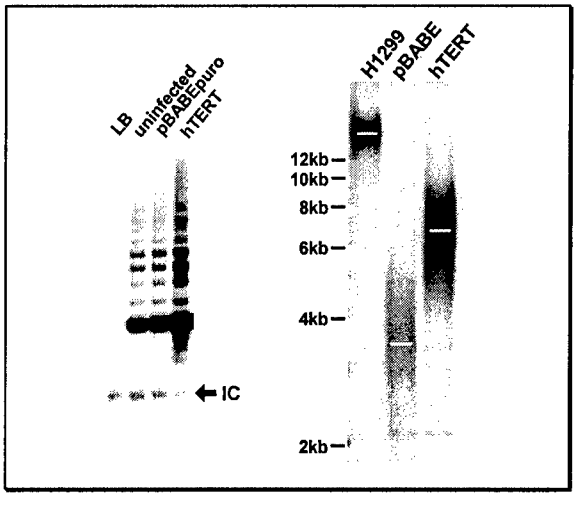

Figure 3. Introduction of hTERT in breast tumor cells (MCF-7) increases telomerase activity and overall telomere length. MCF-7 cells were infected with hTERT and assayed for telomerase activity using the TRAP assay (as in Figure 1) (Left panel). Note that hTERT expressing MCF-7 cells showed a 5-fold increase in activity, even though these breast tumor cells have plenty of telomerase activity already. (Right panel) MCF-7 cells with introduced telomerase have elongated telomere lengths as measured using the TRF assay (Fig. 2).

***Objective #1*: Define the regulation of telomerase by the identification of mammary-specific telomerase interacting proteins using a proteomic approach.**

Our initial experiments have been designed to familiarize ourselves with immunoprecipitating human telomerase using a variety of antibodies, most of which have proven to be useful for precipitating telomerase activity but not for detectable hTERT protein or chaperones (data not shown). We have settled on the 2C4 monoclonal antibody (Novus Biologicals) (Masutomi et al. 2003), which has been shown to effectively detection hTERT by Western and

immunohistochemistry. We have been able to immunoprecipitated hTERT from cells and detect telomerase activity (data not shown). Thus, we have identified the proper antibody for precipitation of telomerase (hTERT) from human cells as a critical first step in this specific aim.

Figure 4 illustrates the protocol for identifying telomerase associated proteins using our cell culture model systems. There are 3 pathways toward the identification of proteins bound to the hTERT molecule after immunoprecipitation: 1-use of 1D gels followed by MALDI-TOF; 2-use of 2D gels and MALDI-TOF; and 3-use of high performance liquid chromatography (HPLC) for protein separation and electrospray mass spectrometry protein detection. The advantage for option #3 is increased sensitivity requiring only femtomole amounts of protein, while the disadvantages are ease of use and specificity (HPLC should provide some separation specificity). With the 2D gels, one should be able to separate and identify bands (spots) very effectively, providing a visual confirmation of precipitated protein. Alternatively, instead of using MALDI-TOF to identify these differentially precipitated proteins, the electrospray mass spec technology could be employed without HPLC. Importantly, since submission of the original grant and even after the June 2004 start date for funding, we have acquired the HPLC/electrospray technology as part of our functional genomics program, which resides on the 5th floor of our building, just down the hall from our laboratories. In addition, the MALDI-TOF has been relocated to the 5th floor as well, so all of the instrumentation needed for this project is easily accessible and usable for personnel in my lab.

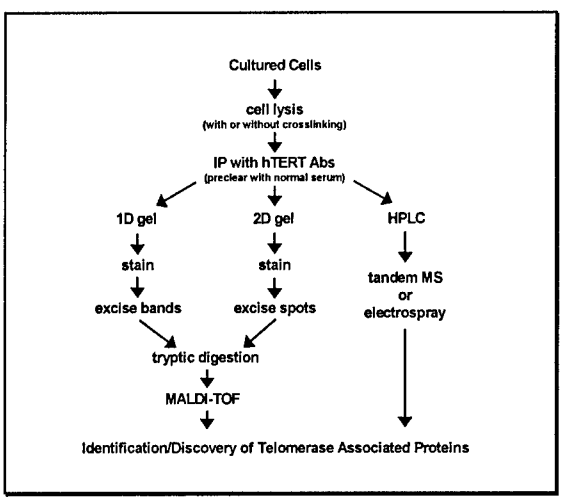

Figure 4. Schematic of the protocol for the discovery of telomerase interacting proteins. The basic design of the experiment is to immunoprecipitate the hTERT telomerase protein subunit from either of the model systems (HME and/or MCF-7 with ectopic telomerase), run the protein on a gel (1D or 2D) or HPLC, and determine the differentially precipitated bands compared to cells with low or no telomerase activity. Chemical cross-linking the proteins may be done prior to IP to increase the detection of associated proteins.

The schematic flowchart in Figure 4 exhibits the 1D gel option, which we have tried extensively after immunoprecipitation of hTERT. Figure 5 shows our best example of a 1D gel using both cell model systems and hTERT immunoprecipitation. Standard coomassie blue staining is not very sensitive for detecting differentially precipitated proteins (top panel – note the 3 bands circled). Yet, we can clearly see more precipitated bands in the visually enhanced form of the gel (use of the Alpha-Innotech gel documentation system to improve the detection of the banding patterns of the gel – lower panel). None of these precipitations utilized crosslinking, which is yet another parameter that is being optimized. In the upper panel of Figure 5, there were 3 bands that were visibly different, and after the more sensitive gel analysis, we found that the 2 bands in the HME 50-5 hTERT lane were in common with all of the cell lines that expressed telomerase,

whether exogenous or endogenous. All 3 of these bands were isolated and gel extracted. These bands were then tryptically digested and subjected to the MALDI. For the 2 bands in the HME 50-5 hTERT lane, they were of sufficient low quantity that no protein was identified. However, the band excised from the MCF-7 lane was found to be actin, suggesting that this was just a background band precipitation. We repeated these experiments and found no differentially precipitated band at 40-45 kD in the MCF-7 cell lines, indicating that the actin band in Figure 5 was nothing more than a spurious result (data not shown).

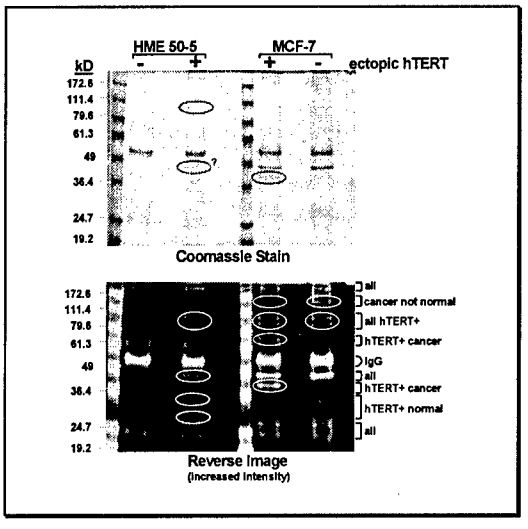

Figure 5. hTERT immunoprecipitation with both ectopic telomerase cell culture models. Cells were immunoprecipitated with hTERT antibodies without crosslinking and electrophoresed on a 1D gel. After staining with Coomassie, differentially precipitated bands were observed and categorized.

Key Research Accomplishments

1- Cell culture models of telomerase over-expression have been created, in both a normal mammary cell strain and a breast tumor cell line.

2-assessed a wide variety of antibodies directed against hTERT for their ability to immunoprecipitate telomerase, settling on a single antibody (2C4 mAb from Novus).

3-identification of differentially precipitated proteins on 1D gel electrophoresis, unfortunately below the level of detection for the MALDI-TOF.

4-Use of telomerase-negative cells as a control allows us to distinguish hTERT associated proteins from those non-specifically associated with the complex.

5-Sub-classification of precipitated bands provides categorization of associated proteins in normal and/or tumor cell lines.

Recommended Changes to the Proposed Work Based on Additional Findings

While we will continue along the proposed experimentation lines, we will likely add to the goals by generating GFP-hTERT MCF-7 and HME cell lines in order to have a visual selectable marker as well as another epitope for precipitation. The use of the GFP-hTERT is important as it has been previously shown that this fusion protein is fully functional, not only in *in vitro* telomerase assays but also in cells by maintaining telomere lengths (Wong et al., 2002). We have made the GFP-hTERT cell line using retroviral system (construction), which provides only modest GFP signal and/or increase in telomerase activity. We are cloning cells and creating more

appropriate vectors (lentiviral constructs) for expression of GFP-hTERT in our cell lines and strains. We will be utilizing positive controls for telomerase binding, proteins already known to associate with hTERT, namely hsp90, p23, and 14-3-3σ. Elimination of gels (i.e. use of HPLC and electrospray technology) may be necessary for ideal detection of telomerase interacting proteins.

Reportable Outcomes

Manuscripts

None related to this project

Abstracts/Presentations

Holt, S.E. DOD Era of Hope, Philadephia, PA. June 2005.

Poynter, K.P., L.W.Elmore, and **S.E.Holt.** DOD Era of Hope, Philadephia, PA. June 2005.

Compton, S.A., L.W.Elmore, and **S.E.Holt.** Telomeres and Telomerase: Cold Spring Harbor Meeting, Cold Spring Harbor, NY. May 2005.

Jensen, K.O., L.W.Elmore, and **S.E.Holt.** Telomeres and Telomerase: Cold Spring Harbor Meeting, Cold Spring Harbor, NY. May 2005.

Jones, K.R., L.W.Elmore, **S.E.Holt**, C.Jackson-Cook, L.F.Povirk, and D.A.Gewirtz. 95th Annual AACR Conference, Orlando, FL, March 2004.

Elmore,L.W., X.Di, C.K.Jackson-Cook, D.A.Gewirtz, and **S.E.Holt.**. Beatson International Cancer Conference: Cell Cycle, Senescence, Apoptosis, and Cancer. Glasgow, Scotland. June 2004.

Holt,S.E., S.A.Compton, K.O.Jensen, and L.W.Elmore. Beatson International Cancer Conference: Cell Cycle, Senescence, Apoptosis, and Cancer. Glasgow, Scotland. June 2004.

Elmore,L.W., X.Di, D.A.Gewirtz, and **S.E.Holt**. BIRCH Annual Meeting, National Institutes of Health, Bethesda, MD, October 2004.

Invited Seminars

Holt,S.E. Eppley Cancer Center, University of Nebraska Medical Center, Omaha, NE. June 2005.

Holt,S.E. Keynote Speaker, The Colorado College Biology Day, Colorado Springs, CO. April 2005.

Holt,S.E. Department of Biology, Maggie Walker Governor's School, Richmond, VA. March 2005.

Holt,S.E. Keynote Speaker, Pugwash Conference, Richmond, VA. March 2005.

Holt,S.E. MD/PhD Program, MCV/VCU, Richmond, VA. December 2004.

Holt,S.E. and S.A.Compton. AACR: Telomeres and telomerase in cancer. San Francisco, CA. November 2004.

Holt,S.E. Mount Desert Island Stem Cell Symposium, MDIBL, Salisbury Cove, ME. August 2004.

Development of Cell Lines

As stated above, we have generated and further characterized MCF-7 breast tumor cells with over-expressed hTERT as well as normal mammary epithelial cells (HMEs) with ectopic telomerase. We are currently generating HME and MCF-7 lines using the GFP-hTERT fusion protein (Wong et al., 2002).

Funding Applied For

none

Conclusions

Upon development of the appropriate cell lines and strains for our proteomic work, we have assayed for telomerase interacting proteins using immunoprecipitation using a variety of hTERT antibodies with limited success for most. A single antibody has proven fruitful and has given some tangible precipitation results, although has not led to the identification of a novel telomerase associated protein. We are currently working on the conditions for fixation of the cells and chemical crosslinking to make certain that associated proteins remain bound to the

telomerase complex during precipitation. In addition, we are perfecting and improving the sensitivity of the 2D gel electrophoresis portion of the proposal in order to effectively utilize the MALDI-TOF for protein identification. In addition, we will use newly acquired methodology/equipment (HPLC followed by electrospray mass spectrometry) The goals for the upcoming year remain to identify novel telomerase interacting proteins, which will provide important clues into defining the regulation of the human telomerase complex. Understanding the regulatory mechanisms related to telomerase in mammary-related cells will facilitate the development of improved therapeutic strategies specifically targeting breast cancer.

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BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Shawn E. Holt, Ph.D.	Associate Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
The Colorado College, Colorado Springs, CO	B.A.	1985-1989	Biology
Texas A&M University, College Station, TX	Ph.D.	1989-1994	Genetics
The University of Texas Southwestern, Dallas, TX	Postdoc	1994-1998	Aging and Cancer

RESEARCH AND PROFESSIONAL EXPERIENCE:, INCLUDING GRANT SUPPORT. DO NOT EXCEED 3 PAGES.

A. Positions and Honors

Positions:

1998-2003	Assistant Professor, Department of Pathology and Department of Human Genetics, Virginia Commonwealth University/Medical College of Virginia, Richmond, VA
2003-present	Associate Professor, Department of Pathology and Department of Human Genetics, Virginia Commonwealth University, Richmond, VA
1998-present	Member, Massey Cancer Center, Virginia Commonwealth University, Richmond, VA
2002-present	Adjunct Faculty, Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA
2002-present	Member, Molecular Biology and Genetics Program, Virginia Commonwealth University, Richmond, VA
2003-present	Director, Graduate Studies and Education, Department of Pathology, Virginia Commonwealth University, Richmond, VA

Honors:

2000-2003	The V Foundation Scholars Program, Cary, NC ($100,000 award)
1996-1998	NRSA Fellowship, National Institute on Aging, while at UT Southwestern, Dallas, TX
1994	Outstanding Presenter, Research Symposium, Texas A&M University, College Station, TX
1994	Outstanding Student Government Member, Texas A&M University, College Station, TX
1988-1989	Dean's List, The Colorado College, Colorado Springs, CO
1988	Most Dedicated Football Player, The Colorado College, Colorado Springs, CO
1987	Rookie of the Year, Baseball, The Colorado College, Colorado Springs, CO
1985-1987	Outstanding College Students of America

B. Selected Peer-Reciewed Publications (over the past 4 years, from a total of 50)

Morales CP, **S.E.Holt**, M.Ouellette, K.Kaur, Y.Yan, K.S.Wilson, M.A.White, W.E.Wright, and Shay JW. 1999. Absence of cancer-associated changes in human fibroblasts immortalized with telomerase. Nature Genetics **21**:55-58.

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Book Chapters (from a total of 4)

Elmore,L.W. and **S.E.Holt**. 2001. Cell Proliferation, Telomerase and Cancer. Advances in Cell Aging and Gerontology: Telomerase, Aging and Disease. Elsevier Science, Inc. pp. 89-102.

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C. Research Support

Active

P.I.: Kennon R. Poynter
Mentor: Shawn E. Holt, Ph.D.
Title: Mechanisms of telomerase inhibition using small inhibitory RNAs and induction of breast tumor cell sensitization
Agency: Department of Defense Breast Cancer program
Amount: $87,000 total direct
Duration: 4/1/04-3/31/07

P.I.: Lawrence F. Povirk, Ph.D. (Co-I, 5% effort, Shawn E. Holt, Ph.D.)
Title: Tyrosyl-DNA phosphodiesterase and oxidative DNA damage
Agency: NIH
Amount: $1,125,000 total direct
Duration: 6/1/04-5/31/09

P.I.: Shawn E. Holt, Ph.D.
Title: Defining the regulation of telomerase through identification of mammary-specific telomerase interacting proteins
Agency: Department of Defense Breast Cancer program
Duration: 3/1/04-2/28/06

Completed

P.I.: Shawn E. Holt, Ph.D.
Title: Mechanisms of Prostate Cancer Transformation
Agency: Department of Defense
Duration: 12/18/01-12/17/04